# THE RELATIONSHIP BETWEEN ANTIOXIDANT ACTIVITY AND LIVER-PROTECTIVE ACTIVITY OF SEVERAL TAIWAN FOLK MEDICINES

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## Abstract

The relationship between antioxidant activity and liver-protective activity against $CCl_4$- induced hepatotoxicity of several Taiwan folk medicines were investigated in this study. Our results have demonstrated that liver-protective effects of Mallotus repandus, Bauhinia championii, Uncaria hirsuta, Uncaria rhynchophylla, Anoectochilus formosanus, Gynostemma pentaphyllum, Ganoderma lucidum, Ganoderma formosanum, Ganoderma neo-japonicum, and Arctium lappa were paralled with the free radical scavenger activity.

Present data have suggested that the potent free radical scavenger activity of these drug extracts may modulate the action of the overload superoxide radical or hydroxyl radical to hepatocytes and reduced the hepatotoxicity caused by $CCl_4$. The result of Bauhinia championii has shown a more potent free radical scavenger activity and liver-protective effect.

Key Words : free radical scavenger, l iver-protective, $CCl_4$, superoxide, hydroxyl radical.

## Introduction

Hepatitis is presently one of the most serious diseases in Taiwan. It is also an important work of the medicinal researcher in Taiwan since viral B hepatitis patients are susceptible to chronic hepatitis, liver cirrhosis, and hepatoma.

An effective therapy is still lacking today, although the development of an effective vaccine has been a major accomplishment. However, it is very popular to treat hepatitis with folk medicines in Taiwan, and the effect is prominent. These crude drugs are highly valued and are worth examining for their potential liver protec-

In recent years, carbon tetrachloride induced liver injury is a well-known experimental liver injury model. During the past year, we have reported in a series of investigation on the effects of several medicinal plants of Taiwan on carbon tetrachloride ($CCl_4$) induced hepatotoxicity (Table 1). However, the mechanism is obscure.

Active oxygen such as superoxide radicals ( $\cdot O_2^-$, $\cdot OOH$) and hydroxyl radicals ( $\cdot OH$) have been indicated to play an important role in the inflammation process during intoxication of ethanol, carbon terachloride and carrageenan (Reddrop et al., 1983; Yoshikawa et al., 1983a, 1983b; Halliwell and Gutteridge, 1984; Yuda et al., 1991). Active oxygens are capable of inducing lipid peroxidation in biological membrances which has been known to occur during intoxication. Experiments using an animal inflammation model show that superoxide scavengers such as superoxide dismutase (SOD) and vitamin E are capable of alleviating inflammation. Based on these findings, the importance of drugs that can eliminate or inhibit the production of active oxygens and lipid peroxide have been realized and such new drugs have lately been developed. In this study, in order to prove the relationship between liver-protective effect and antioxidant effect of crude drug, we examined the effect of above crude drugs extracts on active oxygen scavenging activity by electron spin resonance (ESR) spectrometry using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trapper.

## Materials and Methods

### Plant Materials.

Crude drug samples including Mallotus repandus (Willd) Muell-Arg., ~ABauhinia championii~Z Benth., Uncaria hirsuta Haviland, Uncaria rhynchophylla Miquel, Anoectochilus formosanus Hay., Gynostemma pentaphyllum Makino, Ganoderma lucidum (Fr.) Karst., Ganoderma neo-japonicum Imazeki, Ganoderma formosanum Chang et Chen and Arctium lappa L. were collected from the local market. The indentification of origin was certified by comparative anatomical studies with equivalent specimens preserved in the K.M.C. herbarium of Kaohsiung Medical College, Taiwan. 250 gm of each crude drug was extracted with boiling water (1L × 3). Each extract was filtered, mixed, concentrated and lyophilized.

### Preparation of samples.

100mg of each measurements were put into 10ml 0.1M PBS (sodium phosphate buffer solution) (pH7.8) and dissolved in 40 ℃ for test. To dilute the test solution, pure water was employed.

### Chemicals.

5.5-Dimethyl-1-pyrrdine-1-oxide (DMPO) was supplied by Labotec (Japan).

Diethylenetriamine-pentaacetic acid (DETAPAC) and hypoxanthine (HPX) was obtaind from Sigma, xanthine oxidase (XOD) from Boehringer Mannheim, ferrous sulfate, hydrogen peroxide and L-(+)-ascorbic acid from Wako, while superoxide dismutase (SOD) (3565 unit/mg) from bovine erythrocyte was from Toyobo. The other chemicals used were of the highest grade commercially available.

Superoxide and hydroxyl radicals generating system.

Superoxide radicals were generated from a hypoxanthine-xanthine oxidase reaction (Fenton reaction) system, trapped by DMPO, and the spin adduct (DMPO-OOH) was analyzed using an ESR spectrometer. The procedure was as follows: A solution of 2.0mM HPX (a), 5.5mM DETAPAC (b), various concentration of crude drugs extracts or SOD (c) and 0.4 units/ml XOD (d) was prepared before use. The XOD solution was stored in ice bath to prevent any inactivation of enzyme. 50 $\mu$ l of a, 35 $\mu$ l of b, 50 $\mu$ 1 of c and 15 $\mu$ l of DMPO (9.2M) were put into a test tube. To the mixed solution, 50 $\mu$ l of d was added.

Hydroxyl radicals were generated from the follow procedure : A solution of 1mM ferrous sulfate (a), 5.5mM DETAPAC (b), various concentrations of crude drugs extracts or ascorbic acid (c), and 1mM hydrogen peroxide (d) was prepared before use.

37.5 $\mu$ l of a , 37.5 $\mu$ l of b, 50 $\mu$ l of c and 20 $\mu$ l DMPO (0.092M) were put into a test tube, then 75 $\mu$ l of d was added to the mixed solution. Analysis of spin adduct (DMPO-OH) was performed with an ESR spectrometer.

*ESR spectrometry (Mitsuta et al., 1990; Kohno et al., 1991).*

Reaction mixture was stirred and transferred to an ESR quartz cell (130 $\mu$ l) and placed into the cavity of ESR spectrometer (JEOL-JES-FR80, JEOL Ltd., Tokyo). Forty seconds after the addition of XOD or hydrogen peroxide, the relative intensity of the signal of DMPO-OOH or DMPO-OH spin adduct was measured as the ratio to the internal standard signal of $Mn^{2+}$ in MnO. ESR spectra were recorded at 37 ℃ with a field set 334.7 ± 5.0mT for superoxide radicals and 335.4 ± 5.0mT for hydroxyl radicals, modulation frequency 100KHz, modulation amplitude 0.79 × 0.1mT, response time 0.1s, sweep time 2min, microwave power 8.0mW (9.416 GHz), receiver gain 2 × 100 when superoxide radicals were trapped, and 1 × 100 when hydroxyl radicals were trapped.

## Results and Discussions

Figure 1 & 2 shows the spin adduct DMPO-OOH, formed by HPX-XOD reaction system, and the spin adduct DMPO-OH, formed by ferrous sulfate-hydrogen peroxide reaction system. However, the signal intensities of DMPO-OOH and DMPO-OH

decreased with an increase in the SOD and ascorbic acid concentration as shown in Table 2 & 3. The same results occured in the addition of water extracts of crude drugs. This result can be explained that the reaction between ・O2- and DMPO, ・OH and DMPO can be inhibited by the addition of crude drugs extracts. To investigate the superoxide radicals and hydroxyl radicals scavenger activity of crude drugs, the calibration curves were made. According to the signal intesities, the concentration of extract added, and the calibration curves, the superoxide radicals and hydroxyl radicals scavenger activities were obtained (Table 4).

It has been hypothesized that one of the principal causes of $CCl_4$ induced liver injury is lipid peroxidation by free radical derivatives of $CCl_4$ (Recknagel, 1974). Thus, The protective agents against $CCl_4$ induced liver injury exert their action by impairment of $CCl_4$-mediated lipid peroxidation (I) through inhibited generation of free radical derivatives (Castro et al., 1974; Maling et al., 1974) or (II) due to the antioxidant activity of the protective agent itself (Yasuda et al., 1980).

The scavenger activity shown by the extract demonstrated that a powerful antioxidant could exert a beneficial action against biological and pathological alterations casued by the presence of $CCl_4$, which appears to be metabolized to free radical during intoxication (Hruszkewycz et al., 1978; Noguchi et al., 1982; Recknagel, 1983). B. championii showed the best superoxide radical scavenging activity ($4.71 \times 10^5$ unit/g) and hydroxyl radical scavenging activity (120 unit/g), and these results were confirmed by the liver-protective effect shown on Talbe 1. However, the superoxide radical and hydroxyl radical scavenger activity could partially explain the protective effect of crude drug against $CCl_4$-induced liver injury.

Active oxygen species and free radicals are involved in a variety of pathological events, cancer, and the aging process. In fact, biochemical damage produced by active oxygens, has emerged as a fundamental final common pathway of tissue injury in a wide variety of disparate disease process (Chance et al., 1979; Bulkey, 1983; Dormandy, 1983). Therefore, crude drugs or natural diet food which possess antioxidant or free radicals scavenger activity has become a central focus for research designed to prevent or ameliorate tissue injury and may have a significant role in maintaining health.

The ESR spin-trapping technique is very useful for stabilizing short-lived free radicals. Since this method can be used to analyze crude drugs without any purification, it has therefore been a powerful tool for investigating the scavenging potencies of biological substances contained in crude drugs in terms of their ability to reduce the peaks of spin adducts.

Using this analysis technique we demonstrated that liver-protective effects against $CCl_4$-induced hepatotoxicity of crude drugs mentioned above were paralled with the free radical scavenger activity. This is certainly indication that these crude

drugs contains certain substances which are powerful antioxidant and capable of scavenging superoxide radicals and hydroxyl radicals. Furthermore, since oxygen creates free radicals that impinge on the normal biological functions of organism and lead to cellular aging and the pathogenesis of many age-related diseases, these results may be at least partially attributed to the usefulness in these folk medicine.

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**Table 1. Effect of several Taiwan folk medicines on $CCl_4$ induced hepatotoxicity**

| Drug | GOT | GPT | %protection | References |
|---|---|---|---|---|
| *M. repandus* | 124.32± 5.19[c] | 54.80± 3.05[c] | 100.63 | Lin et al., 1992 |
| *U. rhynchophylla* | 292.54±21.42[b] | 60.56± 6.43[c] | 34.84 | Lin et al., 1992 |
| *U. hirsuta* | 132.58±12.45[c] | 55.90± 3.74[c] | 97.40 | Lin et al., 1992 |
| *B. championii* | 117.98± 5.84[c] | 53.07± 4.95[c] | 103.11 | Lin et al., 1992 |
| *G. pentaphyllum* | 186.55± 9.77[b] | 59.01± 5.73[b] | 57.57 | Lin et al., 1993 |
| *A. formosanus* | 141.98± 4.16[b] | 48.70± 4.75[b] | 84.23 | Lin et al., 1993 |
| *G. lucidum* | 198.13±19.32[b] | 64.37± 5.32 | 45.85 | Lin et al., 1994a |
| *G. neo-japonicum* | 228.73±40.80 | 77.15±20.47 | 24.41 | Lin et al., 1994a |
| *G. formosanum* | 173.08±25.95[b] | 64.68± 9.82[a] | 63.40 | Lin et al., 1994a |
| *A. lappa* | 149.93±14.91[a] | 37.55± 2.88[b] | 54.34 | Lin et al., 1994b |

Signigicantly different from the control, [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$

Table 2.

ESR signal activity of $Mn^{2+}$ and superoxide radical in various concentrations of SOD

| SOD (unit) | ESR Signal Peak Height | | Averaged Relative Peak Height |
|---|---|---|---|
| | $Mn^{+2}$ | Radical | |
| 0.000 | 84.800 | 203.000 | 2.394 |
| 1.850 | 85.800 | 165.600 | 1.930 |
| 4.625 | 79.400 | 112.400 | 1.416 |
| 9.250 | 81.800 | 74.400 | 0.910 |
| 13.875 | 86.800 | 57.600 | 0.655 |
| 18.500 | 82.000 | 47.400 | 0.547 |

Calibration curve: Y=0.193756X-0.145256 R=0.998457

Where $Y = \left[\frac{I_0}{I} - 1\right]$, X= [SOD (unit)]

I indicates the averaged relative peak height in various concentrations of SOD

Table 3.

ESR signal activity of $Mn^{2+}$ and hydroxyl radical in various concentration of Ascorbic acid.

| Ascorbic acid (mM) | ESR Signal Peak Height | | Averaged Relative Peak Height |
|---|---|---|---|
| | $Mn^{2+}$ | Radical | |
| 0.00 | 45.60 | 514.60 | 11.31 |
| 0.20 | 45.40 | 417.90 | 9.21 |
| 0.40 | 44.40 | 293.90 | 6.62 |
| 0.60 | 46.00 | 200.40 | 4.36 |
| 0.80 | 46.80 | 124.00 | 2.65 |

Calibration curve: $Y=0.05921X^3-0.36267X^2+0.79380X+0.01279$

where $Y=[AsA\ (mM)]$, $X=\left[\frac{Io}{I}-1\right]$

*I indicates the averaged relative peak height in various concentration of Ascorbic acid (AsA)

Table 4. Superoxide and hydroxyl radical scavenging effect of several Taiwan folk medicines

| Drug | [a]Superoxide radical scavenging activity (unit/g) | [b]Hydroxyl radical scavenging activity (unit/g) |
|---|---|---|
| *M. repandus* | $2.69 \times 10^5$ | 108 |
| *U. rhynchophylla* | $1.49 \times 10^5$ | 51 |
| *U. hirsuta* | $4.93 \times 10^4$ | 40 |
| *B. championii* | $4.71 \times 10^5$ | 120 |
| *G. pentaphyllum* | $3.33 \times 10^3$ | 41 |
| *A. formosanus* | $8.85 \times 10^3$ | 63 |
| *G. lucidum* | $6.28 \times 10^3$ | 43 |
| *G. neo-japonicum* | $8.52 \times 10^3$ | 47 |
| *G. formosanum* | $1.74 \times 10^4$ | 97 |
| *A. lappa* | $2.00 \times 10^3$ | 46 |

a. The unit was designed by J.M. McCord and I. Fridovich [J. Biol. Chem., 244, 6049 (1969)]

b. I unit means the hydroxyl radical scavenging activity by 1mM ascorbic acid in this Fenton reaction system.

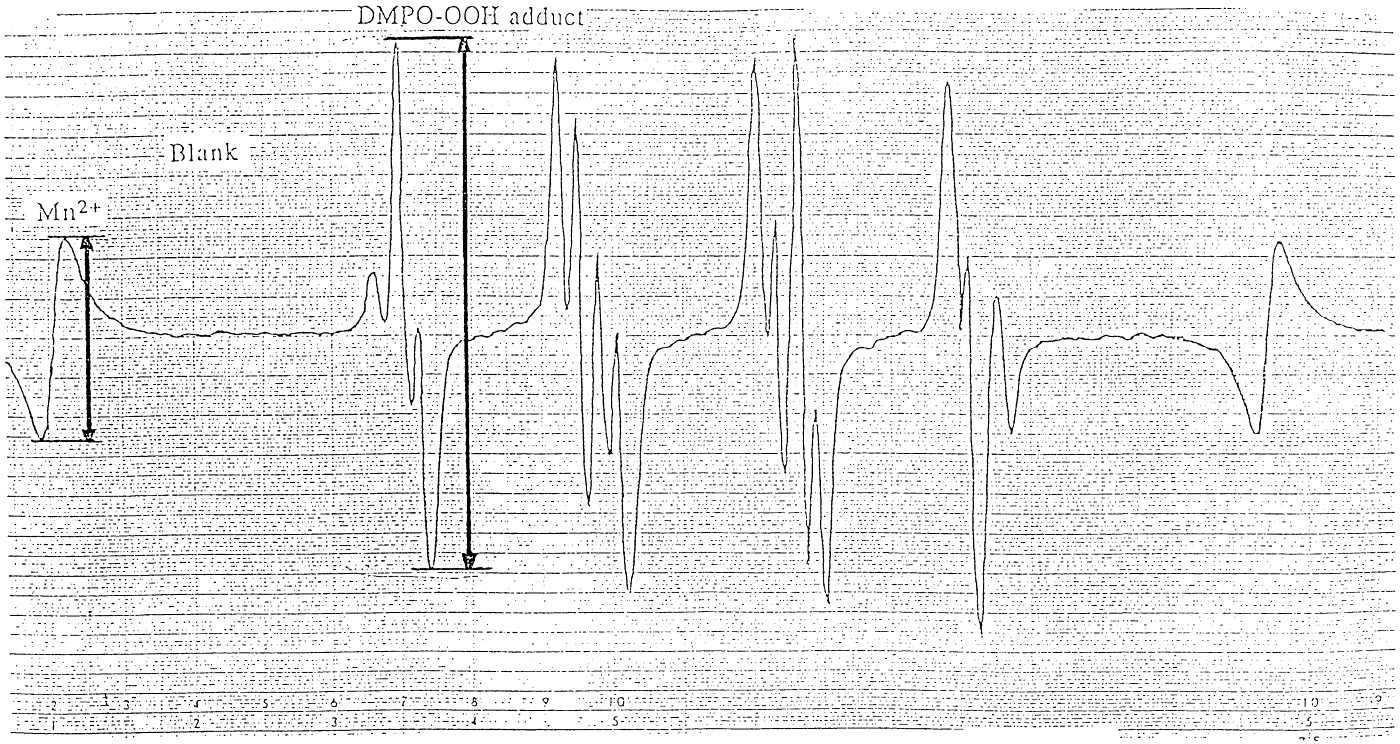


Fig.1. ESR signals of superoxide radical from DMPO-OOH adduct.

$Mn^{2+}$ : manganese oxide signal as a standard.

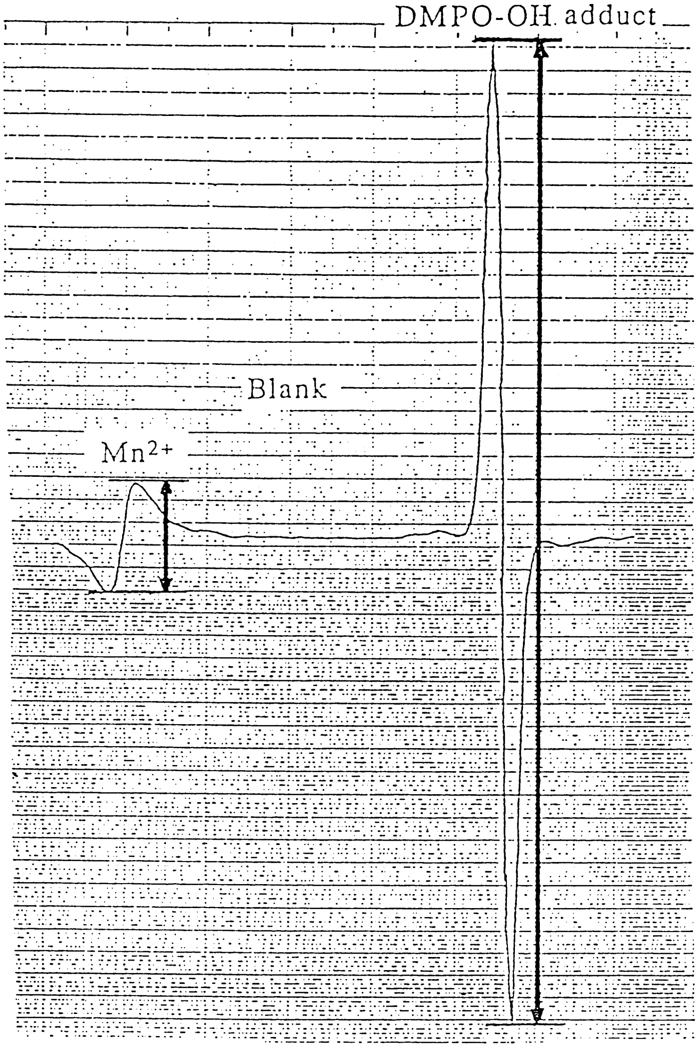


Fig.2. ESR signals of hydroxyl radical from DMPO-OH adduct.
$Mn^{2+}$ : manganese oxide signal as a standard.

# 摘 要

本次研究主要探討台灣數種常用保肝生藥，其對四氯化碳所誘發之急性肝炎的防治效果與生藥抗氧化活性之關係。研究結果證實保肝生藥，包括 Mallotus repandus, Bauhinia championii, Uncaria hirsuta, Uncaria rhynchophylla, Anoectochilus formosanus, Gynostemma pentaphyllum, Ganoderma lucidum, Ganoderma formosanum, Ganoderma neo-japonicum 及 Arctium lappa 確實具有清除自由基的抗氧化能力。

此次結果亦顯示，具有強力清除自由基能力的生藥能夠改善由於過量活性氧，包括 superoxide radical 及 hydroxyl radical 對肝細胞之傷害，並減輕四氯化碳所導致之肝毒性。此次研究之生藥中 Bauhinia championii 則顯示了最佳之保肝作用及最強的自由基清除活性。